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Endothelial pentraxin 3 contributes to murine ischemic acute kidney injury

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Toll-like receptor 4 (TLR4), a receptor for damage-associated molecular pattern molecules and also the lipopolysaccharide receptor, is required for early endothelial activation leading to maximal inflammation and injury during murine ischemic acute kidney injury. DNA microarray analysis of ischemic kidneys from TLR4-sufficient and -deficient mice showed that pentraxin 3 (PTX3) was upregulated only on the former while transgenic knockout of PTX3 ameliorated acute kidney injury. PTX3 was expressed predominantly on peritubular endothelia of the outer medulla of the kidney in control mice. Acute kidney injury increased PTX3 protein in the kidney and the plasma where it may be a biomarker of the injury. Stimulation by hydrogen peroxide, or the TLR4 ligands recombinant human high-mobility group protein B1 or lipopolysaccharide, induced PTX3 expression in the Mile Sven 1 endothelial cell line and in primary renal endothelial cells, suggesting that endothelial PTX3 was induced by pathways involving TLR4 and reactive oxygen species. This increase was inhibited by conditional endothelial knockout of myeloid differentiation primary response gene 88, a mediator of a TLR4 intracellular signaling pathway. Compared to wild-type mice, PTX3 knockout mice had decreased endothelial expression of cell adhesion molecules at 4 h of reperfusion, possibly contributing to a decreased early maladaptive inflammation in the kidneys of knockout mice. At 24 h of reperfusion, PTX3 knockout increased expression of endothelial adhesion molecules when regulatory and reparative leukocytes enter the kidney. Thus, endothelial PTX3 plays a pivotal role in the pathogenesis of ischemic acute kidney injury.

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Although ischemic acute kidney injury (AKI) continues to have a high incidence and mortality despite modern supportive therapy, and leads to progressive kidney disease that has its own high mortality,[1–3] the pathogenesis remains poorly understood.

Toll-like receptor 4 (TLR4) is required for the inflammatory response that exacerbates the initial ischemic injury.[4] We previously showed that HMGB1 released by injured renal cells bound endothelial TLR4, and this increased the expression of proinflammatory adhesion molecules.[5] In the absence of endothelial TLR4, these adhesion molecules were not expressed, inflammation was decreased, and injury ameliorated. These data incriminate TLR4 as the trigger for the initial endothelial activation necessary for inflammation and maximal injury during ischemic injury. To better understand the maladaptive role of TLR4, we compared genome-wide gene expression at 4 h of reperfusion in kidneys from wild-type (WT) C57BL/10 mice vs. TLR4-null C57BL/10ScNJ mice using Affymetrix GeneChip Mouse Genome 430 2.0 Array chips. One of the most differentially expressed genes was pentraxin 3 (PTX3; see NCBI Gene Expression Omnibus (Edgar), GEO Series accession number GSE34351 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34351)).

We now report that knockout (KO) of PTX3 ameliorates ischemic AKI. PTX3 is the prototypic member of the long pentraxin family that is produced in peripheral tissues. It is conserved from arachnids to humans. In particular, its gene organization, structure, and promoter are highly conserved in humans and mice. This not only suggests the fundamental importance of PTX3 in biology and disease, but also that translation of murine studies to human disease should be possible.[6] Although PTX3 increases in human plasma after acute ischemia–reperfusion injury (IRI) to the brain and heart, and although this increase is proposed as a clinically useful and early reliable prognostic marker for bad outcomes,[7,8] whether and how PTX3 contributes to pathophysiology of IRI is not well established.

Altogether, the above data suggest fundamental important links between TLR4, endothelium, and PTX3 in the

pathophysiology of renal AKI. We now explore these links for the first time experimentally. We localize PTX3 to renal endothelium, explore its regulation by reactive oxygen species (ROS), TLR4, and the Myd88-dependent signaling pathway of TLR4, and determine the effect of PTX3 KO on endothelial functions. Our data establish a maladaptive role for PTX3 during ischemic AKI.

RESULTS

PTX3 KO attenuates ischemic AKI

We compared the response of WT vs. PTX3 homozygous KO (PTX3 KO) kidneys to IRI caused by temporarily occluding the renal pedicle (see 'Materials and Methods'). At 24-h reperfusion, both measures of renal function increased significantly in WT mice compared with PTX3 KO mice (Figure 1a and b). For sham-operated mice, the renal function remained similar and close to baseline in both strains (data not shown).

Note that we used capillary electrophoresis to measure the serum creatinine (Scr).[9] This is more accurate than the usual Jaffe method, which is confounded by extraneous chromophores in murine serum. Thus, values obtained by direct chemical measurements (HPLC and capillary electrophoresis) are 1/6th of the values obtained by the Jaffe-type measurements.[10,11] A rise of the Scr from 0.1 to 0.4 mg/dl in our assay represents significant renal injury. In Figure 1, we used an ischemia time of 16 min that allowed all the WT mice to survive for the entire 7-day experiment. We observed the same protection by PTX3 KO after a 23-min ischemia time, but some WT mice died. In most experiments in which kidneys were harvested at 4 h and survival of the WT beyond 24 h was not important, we used the 23-min ischemia time because this gave a larger signal for PTX3 and the other proinflammatory molecules in the WT groups.

We also analyzed the histology of the kidneys by scoring the tubular damage and inflammation. After IRI, we observed less injury and inflammation in PTX3 KO ischemic kidneys. Kidney injury scores were significantly more severe in the WT compared with PTX3 KO kidneys (Figure 1c and d). To study leukocyte infiltration, we stained kidney sections with myeloperoxidase and counted positively staining cells per 10 high-power fields. WT kidneys had significantly more inflammation (Figure 1c and e).

PTX3 is increased in ischemic WT kidneys but not in TLR4 KO kidneys

We found that IRI increased PTX3 protein in WT kidneys by ELISA. At 4-h reperfusion, PTX3 increased by 2.1-fold compared with sham controls (14.0 ± 1.1 vs. 6.5 ± 0.6 ng/ml). It reached a peak value of 45 ± 2.5 ng/ml on day 1 (vs. sham 16.0 ± 1.1 ng/ml) and then gradually dropped down to baseline by day 7 (Figure 2a). In addition, we found a significant elevation of plasma PTX3 at 4-h reperfusion (96 ± 5.6 vs. 60 ± 4.2 ng/ml), which peaked at 24-h reperfusion (265 ± 9.8 vs. 202 ± 7.3 ng/ml; Figure 2b). This increase is similar to increases seen after ischemic injury of other organs—brain after stroke and myocardial ischemia.[7,8] Because PTX3 is produced at the site of injury,[6] the plasma PTX3 is thought to enter the blood from the injured brain, heart, or, in our experiment, kidney. The increased plasma PTX3 in the sham mice may represent PTX3 produced by skin, muscle, and connective tissues that are injured by the surgery necessary to expose and then place the vascular clamp across (IRI) or beneath (sham) the renal arteries.

The increase in plasma PTX3 at 4-h reperfusion may be particularly important. See points enclosed in box in Figure 2b. This increase occurs in the IRI group well before the increase in the sham group, and well before the increase in Scr in our AKI model. This suggests that PTX3 may be a biomarker for the early detection of AKI. The search for such biomarkers, which appear before an increase in Scr, is a major ongoing effort in nephrology.[12]

We previously showed the importance of endothelial TLR4 in the pathogenesis of ischemic AKI. DNA microarray data suggested that PTX3 increased on WT ischemic kidneys. Further quantitative reverse transcription PCR confirmed that IRI increased renal PTX3 and ESM1 (endothelial cell–specific molecule 1, or endocan) messenger RNA by 8.27 ± 0.75-fold and 3.35 ± 0.55-fold, respectively, in WT kidneys. However, TLR4 KO prevented such increases (Figure 3a and b).

We also compared PTX3 protein expression in sham kidneys with that in ischemic WT kidneys by immunohistology. Consistent with the increase in PTX3 messenger RNA, Figure 3c shows increased peritubular PTX3 protein in the outer medulla at 4-h reperfusion in WT kidneys. In the TLR4 KO mice, we did not see such increases. Supplementary Figure S1 online shows that the anti-PTX3 antibody was specific because there was negligible staining of ischemic PTX3 KO kidneys.

PTX3 is expressed predominantly on renal endothelia

As PTX3 was upregulated early during AKI, we performed immunostaining on frozen sections at 4-h reperfusion. In the sham kidney harvested 4 h after reperfusion, we found PTX3 on peritubular structures of the outer medulla (Figure 4a). In addition, there was scattered rare peritubular staining in the cortico–medullary junction, and we did not detect PTX3 on glomeruli (Figure 4a). No PTX3 was seen in the inner medulla (not shown). We localized PTX3 to peritubular endothelia by double staining with PTX3 and the endothelial marker CD31 (Figure 4b and Supplementary Figure S2 online). Previous studies in the kidney after IRI confirmed CD31 as an appropriate marker of endothelia.[13,14] At 4 h after IRI, PTX3 increased on the kidney. There was new expression of PTX3 on glomerular endothelia and on inner medulla in addition to increased expression in the outer medulla (Figure 4c). For high resolution photomicrographs of Figure 4, please see Supplementary Figure S5 online.

In addition to immunohistology, we developed techniques to isolate renal endothelia or leukocytes using beads coated with specific antibodies for endothelial cells or leukocytes. This technique, validated in our previous publication,[5] confirmed that over 95% of PTX3 was on endothelia

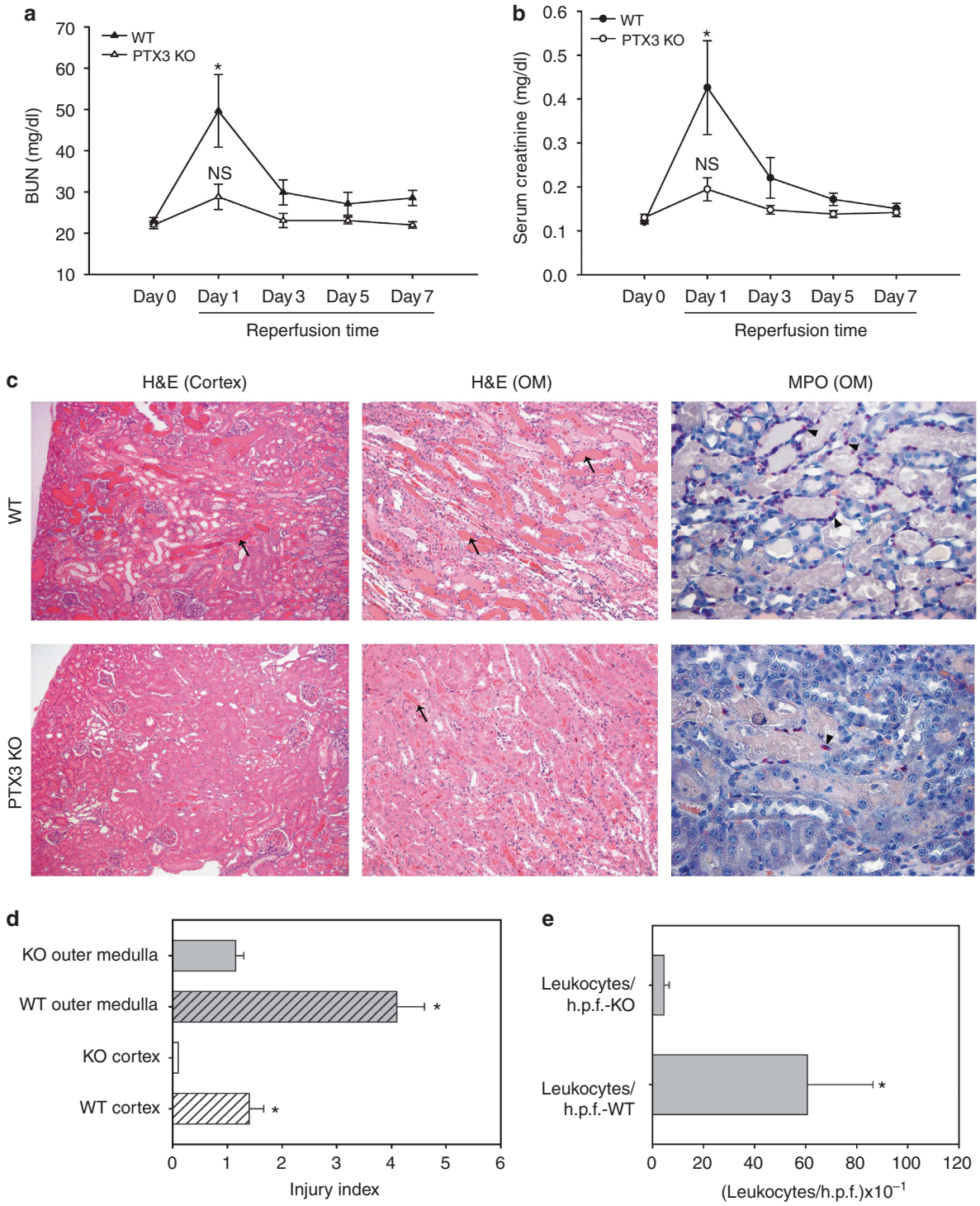

Figure 1 | Pentraxin 3 (PTX3) deficiency ameliorates ischemic acute kidney injury (AKI). Renal pedicles of PTX3 knockout (KO) mice and wild-type (WT) littermates were clamped for 16 min. Blood samples were collected on days 1, 3, 5, and 7 reperfusion. Day 0 indicates samples from mice without operation. (**a**) Blood urea nitrogen (BUN) was measured using a colorimetric method. (**b**) Serum creatinine was measured using a capillary electrophoresis method. Error bars represent mean ± s.e.m., $n = 5$ per group, $^*P < 0.05$ WT day 1 vs. day 0; NS, not significant PTX3 KO day 1 vs. day 0. (**c**) Hematoxylin and eosin (H&E) and myeloperoxidase (MPO) staining: at 24-h reperfusion, kidneys were harvested and fixed in formalin. H&E and MPO stainings were performed on paraffin sections. Arrow shows one of many damaged tubules. Original magnification, × 10. Arrowhead indicates one of many positively staining cells for MPO (× 20). (**d**) Injury index at 24-h reperfusion: tissue damage was scored in the cortex and outer medulla (OM). (**e**) Leukocyte infiltration at 24-h reperfusion. The number of inflammatory cells in cortex and OM was counted. In both **d** and **e**, error bars represent mean ± s.e.m., $n = 5$ per group, $^*P < 0.05$ WT vs. KO. h.p.f., high-power field.

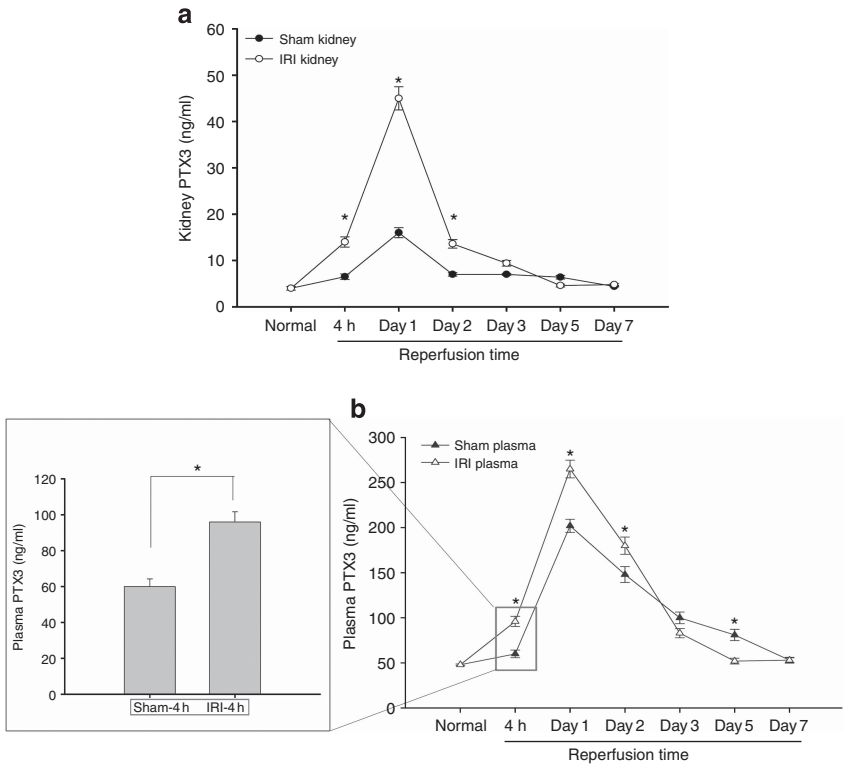

Figure 2 | Pentraxin 3 (PTX3) protein increases in kidney and plasma during acute kidney injury (AKI). Renal pedicles of wild-type mice were clamped for 16 min. Kidney samples were collected from normal kidneys and ischemic kidneys harvested at 4 h, day 1, day 3, day 5, and day 7 reperfusion. Tissues were disrupted using a homogenizer in RIPA buffer supplemented with protease inhibitors. Kidney protein concentration was determined by the Bradford assay in triplicate, and the same amount of protein from each kidney was assayed. Plasma and kidney PTX3 levels were measured using the Quantikine PTX3 immunoassay kit from R&D Systems. (**a**) Time-course study of kidney PTX3. Error bars represent mean ± s.e.m., $n = 4$, $^*P < 0.001$ compared with sham at each time point. (**b**) Time-course study of plasma PTX3. Error bars represent mean ± s.e.m., $n = 4$ per group, $^*P < 0.01$ vs. sham at the same time point. Plasma PTX3 at 4-h reperfusion was highlighted in rectangle, and shown in the box. IRI, ischemia-reperfusion injury.

(Supplementary Figure S3A and Supplementary Text online). Furthermore, our *in vitro* studies, discussed in the next section, also support the expression of PTX3 on endothelia.

Endothelial PTX3 is regulated by TLR4 and ROS

We studied the regulation of PTX3 in the MS1 endothelial cell line, primary renal endothelial cultures (ECs) from WT mice, and mice with conditional KO of MyD88 on endothelia.

Regulation of PTX3 by TLR4 and ROS in MS1 endothelial cells in vitro. HMGB1 and ROS are potential signals for endothelial PTX3 production during ischemic AKI. Injured and dying renal cells release intracellular HMGB1 into the extracellular space where it has proinflammatory properties.[15] We previously demonstrated that HMGB1 ligated endothelial TLR4 during ischemic AKI and induced the expression of endothelial adhesion molecules necessary for maladaptive inflammation.[5] In addition to HMGB1, ROS is produced during ischemic AKI and elicits maladaptive responses.[16–18] Figure 5a shows that recombinant human high-mobility group protein B1 (rhHMGB1) increases PTX3 expression by MS1 cells *in vitro* by 1.6 ± 0.3-fold. As a positive control, we found that MS1 cells also responded by 1.9 ± 0.4-fold to ultrapure lipopolysaccharide (LPS) that is the exogenous ligand for TLR4. To test the hypothesis that endothelial cells will produce more PTX3 under oxidative stress, we treated MS1 cells with H_2O_2 at 100 µmol/l for 30 min and replenished cells with complete medium for 4 h *in vitro*. This is to mimic the *in vivo* IRI scenario. H_2O_2 induced PTX3 on MS1 by 5.0 ± 1.2-fold (Figure 5b). Figure 5c shows that rhHMGB1 or LPS enhanced the PTX3 expression induced by H_2O_2.

PTX3 is regulated by TLR4 and ROS in primary renal endothelial cell cultures. The MS1 endothelial cells mentioned above were originally derived from mouse pancreas,[19,20] and may not accurately reflect renal endothelial cells. We therefore developed techniques to isolate renal endothelial cells and study their PTX3 expression. We isolated CD31+ cells from renal digests and established a primary EC culture. When put into EC culture medium, CD31+ cells started to grow into small cell clusters within 3 days. By day 8, cells would reach confluence and had the signature 'cobblestone' appearance of endothelial cells (Figure 6a). We did not notice any growth difference between cells harvested from normal kidneys or ischemic kidneys. We used passage 1 for our experiment.

Figure 6b showed that PTX3 was upregulated in the presence of H_2O_2 (1.5 ± 0.2-fold), rhHMGB1 (1.7 ± 0.2 fold),

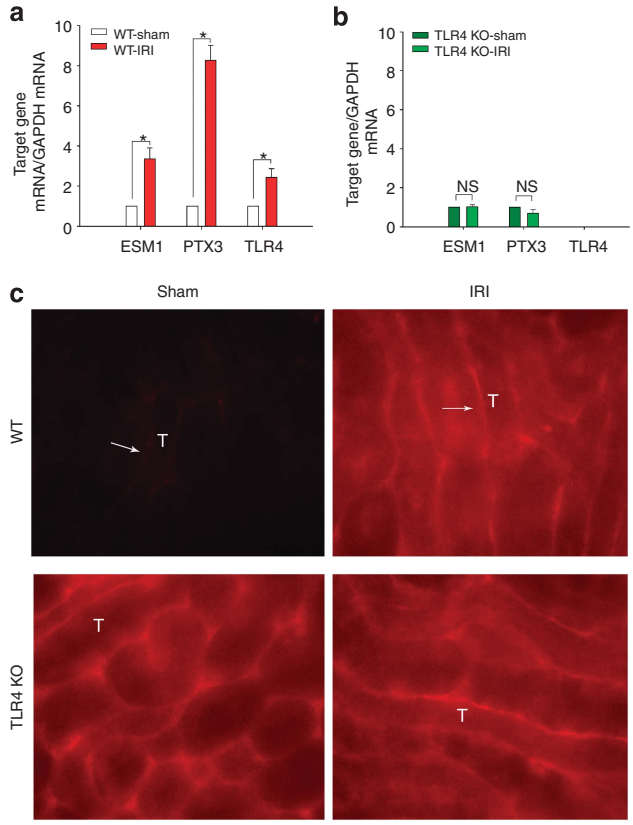

Figure 3 | Toll-like receptor 4 (TLR4) is required for increased pentraxin 3 (PTX3) in acute kidney injury (AKI). Renal pedicles of wild-type (WT) B10 and TLR4 knockout (KO) mice were clamped for 23 min and kidneys harvested at 4-h reperfusion. The genes of interest (PTX3, EsM1, and TLR4) were determined by quantitative reverse transcription PCR and analyzed by the comparative *C*t method. The calibrator gene is the gene of interest taken from the sham kidney. (**a**) WT kidneys. (**b**) TLR4 KO kidneys. Error bars show mean ± s.e.m., $n = 6$ in each group, $^*P < 0.01$ ischemia-reperfusion injury (IRI) compared with sham; NS, not significant. (**c**) Immunohistology shows increased PTX3 in WT kidneys at 4-h reperfusion. A rat anti-mouse PTX3 monoclonal antibody was used to stain frozen sections from paraformaldehyde-fixed tissues. Exactly the same staining conditions and exposures were used to compare PTX3 expression in sham and AKI kidneys. PTX3 is located on peritubular capillaries of the outer medulla. PTX3 was increased on WT ischemic kidneys. No increased endothelial PTX3 was found on ischemic TLR4 KO kidneys (× 40). Arrows indicate some of many capillaries positive for PTX3; 'T' indicates a few of many tubules. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

or LPS (1.9 ± 0.3 fold). Combined treatment of H_2O_2 and rhHMGB1 (2.1 ± 0.1 fold), or H_2O_2 and LPS (3.6 ± 0.2 fold), induced a much higher level of PTX3.

MyD88 conditional KO on the endothelium decreases PTX3 expression and other markers of endothelial activation. Our observations above suggest that PTX3 is regulated, in part, by TLR4. To better understand this regulation, we examined PTX3 expression with a conditional endothelial MyD88 KO mouse. MyD88 is required for one of the two major pathways of TLR4 intracellular signaling, and contributes to the pathogenesis of ischemic AKI.[21–23] To delete MyD88 on endothelial cells ('conditional MyD88 KO'), we used an established mouse with floxed MyD88[24] and the Tie2Cre mouse[25,26] that expresses cre-recombinase uniquely on endothelia during ischemic AKI.[13,14] The MyD88 KO mouse is normal, except in its responses to infection and injury.[27] Baseline renal and plasma PTX3 levels were similar in the 'conditional MyD88 KO' and MyD88 f/f; Tie2Cre(−) ('WT') mice. Using this cre-lox strategy, we were able to knock out MyD88 from renal endothelial cells by 50%. Despite this substantial residual endothelial MyD88, we found dramatic phenotypes with regard to renal and plasma PTX3 protein after IRI, as well as in endothelial activation markers.

The main point of these studies was that at 18-h reperfusion there was much less renal PTX3 protein in the 'conditional MyD88 KO' kidneys (21.9 ± 2.3 ng/ml) than in 'WT' kidneys (33.8 ± 2.8 ng/ml); see Figure 7a. This decreased PTX3 in ischemic 'conditional MyD88 KO' kidneys was associated with decreased plasma PTX3 at 18-h reperfusion (341.0 ± 12.2 ng/ml) compared with 'WT' plasma (480.0 ± 15.9 ng/ml); see Figure 7b. The decreased plasma PTX3 confirms the decreased production in the 'conditional KO' kidneys. These studies suggest that endothelial PTX3 is regulated by the endothelial MyD88-dependent pathway of TLR4 signaling during ischemic AKI.

There was a much smaller increase in renal and plasma PTX3 after sham surgery in both 'conditional MyD88 KO' and 'WT' mice. Because this increase was the same in both types of mice, we believe that it was independent of endothelial Myd88 and was due to cytokine released from the surgical trauma to skin and muscle that was common to both groups of mice and necessary to expose the renal pedicle. Cytokines such as tumor necrosis factor-α are known to regulate PTX3 production.[6] These results are consistent with the idea that the uninjured kidney responds to cytokines produced by injured distant tissues.[28]

Another major point of Figure 7 is the inhibitory effect of 'conditional MyD88 KO' on endothelial adhesion molecules. In WT kidneys, such adhesion molecules are required for the inflammatory response to IRI that exacerbates injury.[5] Figure 7c shows that after IRI, endothelial cells isolated from 'WT' endothelia increased their expression of ICAM1 (2.3 ± 0.2-fold), VCAM1 (2.3 ± 0.8-fold), E-selectin (3.5 ± 0.3-fold), and P-selectin (2.2 ± 0.1-fold). In contrast, endothelia isolated from 'conditional MyD88 KO' mice did not increase these adhesion molecules. Furthermore, 'WT,' but not 'conditional MyD88 KO,' endothelia increased their expression of ESM1 (5.9 ± 0.4-fold), which is uniquely found on endothelia,[29] NOS3 (3.5 ± 0.2-fold), and fibroblast growth factor 2 (FGF2) (3.9 ± 0.3-fold). Note that ESM1 expression after IRI was also decreased in TLR4-deficient kidneys (Figure 3). All of the three molecules are thought to ameliorate ischemic AKI.[29–33] Finally, to our knowledge, these are the first studies to demonstrate increased expression of ESM1, NOS3, FGF2, CXCL4, and CXCL1 on renal endothelia isolated after IRI.

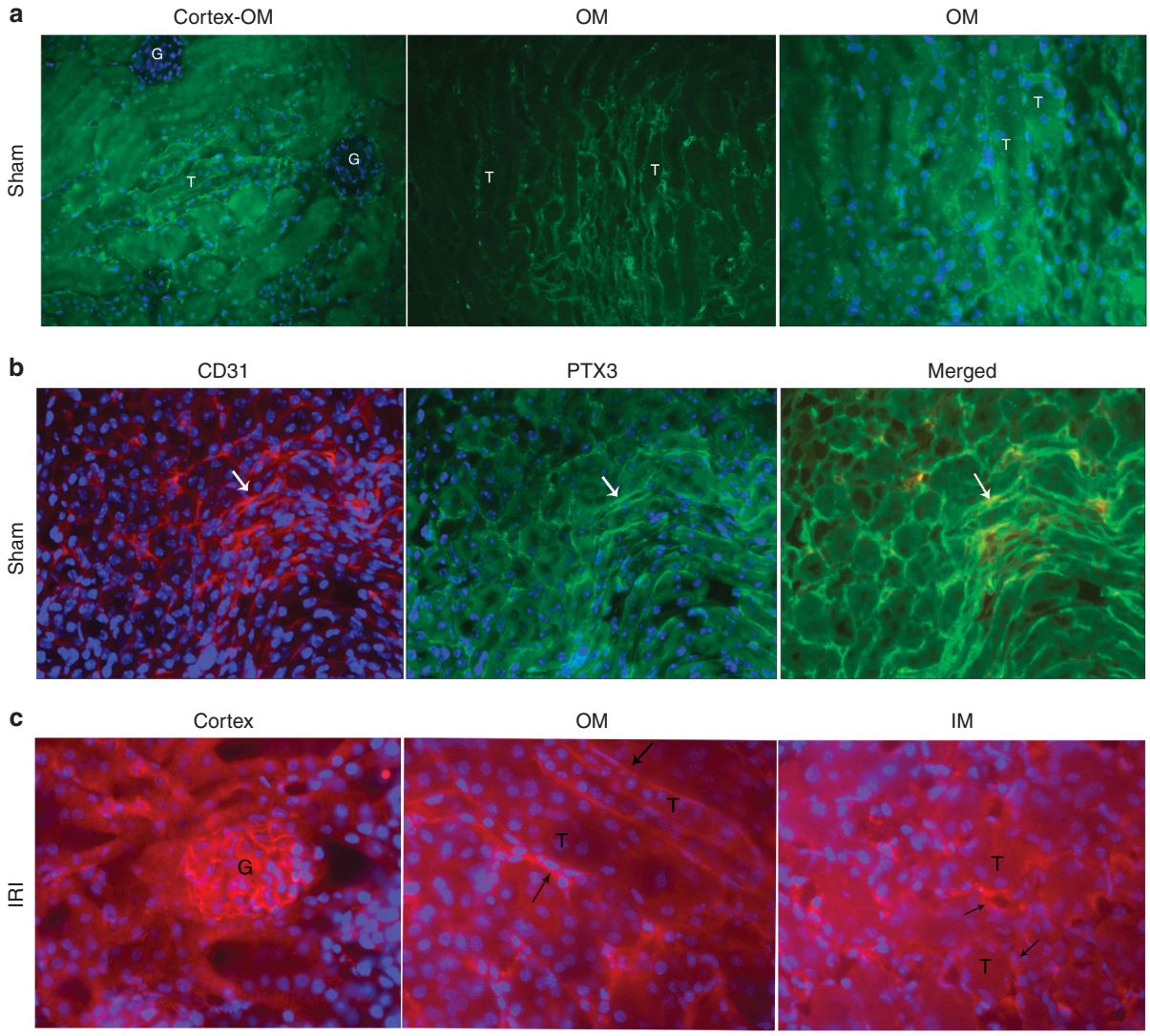

Figure 4 | Pentraxin 3 (PTX3) colocalizes with CD31 + cells on kidney. Renal pedicles of wild-type (WT) mice were clamped for 23 min. Kidney was *in situ* perfused with 4% paraformaldehyde at 4 h after reperfusion and then snap-frozen. (**a**) In sham kidney at 4-h post operation, PTX3 was detected on the peritubular structure in the outer medulla (OM). Representative staining of PTX3 in the conjunction of cortex–OM area (× 10), OM area (× 20), and OM area (× 40). (**b**) PTX3 colocalized with CD31 on the kidney section. A rabbit polyclonal anti-PTX3 was used in **a** and **b**. In **b**, a rat monoclonal antibody was also used to stain for CD31 (× 20), and species-specific secondary antibodies allowed double staining for PTX3 and CD31. (**c**) PTX3 was detectable only by a rat monoclonal antibody after ischemia-reperfusion injury (IRI). At 4-h reperfusion, PTX3 was expressed on glomeruli, peritubular capillaries in both OM and inner medulla (IM; × 20). Arrows pointed to endothelia; 'T', tubules, 'G', glomeruli. Each stain was carried out on four individual mice per group, and the images in the figures are representative of each group. See Supplementary Figure S5 for high resolution photomicrographs.

PTX3 KO differentially changes endothelial function at 4 vs. 24 h after renal IRI

The data given below show that PTX3 KO profoundly alters the biology of renal endothelial cells during ischemic AKI. This may explain how PTX3 KO ameliorates this disease.

Using anti-CD31-conjugated Dynabeads, we isolated and compared renal endothelial from WT vs. PTX KO mice at 4-h and 24-h reperfusion. We found that PTX3 KO had opposite effects at these two time points.

At 4-h reperfusion, inflammation exacerbates ischemic injury.[34,35] We previously showed that at this time WT endothelia increased their expression of adhesion molecules (ICAM1, VCAM1, E-selectin), and that TLR4 KO prevented maladaptive inflammation by preventing such adhesion molecule expression.[5] Figure 8a shows that PTX3 KO also prevented the expression of these adhesion molecules. We also examined other endothelial activation markers. Similar to the conditional MyD88 KO (Figure 7c) and unlike the WT endothelial cells, PTX3 KO did not increase the expression of ESM1 or NOS3. These data suggest that PTX3 is downstream of TLR4, and that the expression of these adhesion molecules is downstream of PTX3.

At 24-h reperfusion, inflammation may have a different function. Instead of exacerbating injury, late inflammation

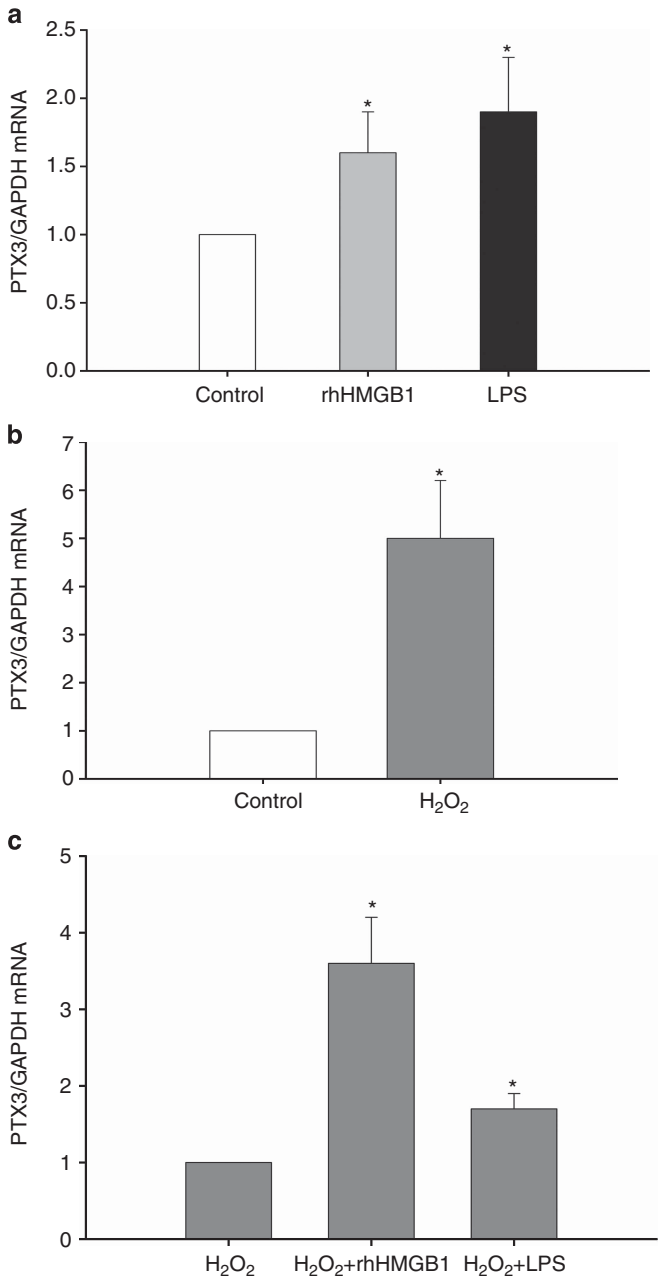

Figure 5 | Toll-like receptor 4 ligands and reactive oxygen species increase pentraxin 3 (PTX3) expression in the MS1 cell line. MS1 cells were cultured in Dulbecco's modified Eagle's medium + 10% fetal calf serum until reaching confluence. Cells were stimulated with (**a**) recombinant human high-mobility group protein B1 (rhHMGB1) or lipopolysaccharide (LPS) at 5 μg/ml for 4 h, or (**b**) H_2O_2 at 100 μmol/l in Earle's balanced salt solution for 30 min and then replenished with complete medium for 4 h, or (**c**) H_2O_2 at 100 μmol/l for 30 min and followed by rhHMGB1 or LPS (5 μg/ml) in complete medium for 4 h. PTX3 messenger RNA (mRNA) was measured from harvested cells by quantitative reverse transcription PCR. Error bars show mean ± s.e.m., $n = 5$, $^*P < 0.05$ compared with the control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

may inhibit aggressive leukocyte activity, and reparative macrophages may facilitate repair.[36–40] In contrast to the 4-h reperfusion time point when PTX3 KO endothelial cells decreased adhesion molecule expression, Figure 8b shows the PTX3 KO increased expression of the adhesion molecules ICAM1, VCAM1, and P-selectin. This suggests that PTX3 KO endothelia may facilitate immigration of regulatory and reparative leukocytes into the injured kidney.

PTX3 KO alters renal endothelial function in primary cultures

To further explore the effect of PTX3 KO on the renal endothelium, we developed techniques to isolate and then culture primary endothelial cells from WT vs. PTX3 KO kidneys. These primary ECs were then stimulated with the TLR4 ligands HMGB1 or LPS. As opposed to the endothelia directly isolated from sham and ischemic kidneys in Figure 8 above, these primary endothelial cells have not been injured by IRI *in vivo*, and have been in the presence of growth factors vascular endothelial growth factor (VEGF), FGF, insulin-like growth factor 1 (IGF1), hepatocyte growth factor 1 (HGF1), hydrocortisone, and heparin) that facilitate the survival of these cells *in vitro* and attempt to mimic the *in vivo* microenvironment. The main point learned from these primary cultures is that renal endothelia from PTX KO vs. WT mice are fundamentally different.

Three different groups of endothelial responses were seen (Figure 9). The first group (ICAM-1, VCAM 1, E-selectin) of responses were the same in WT and PTX3 KO endothelial cells. These are proinflammatory adhesion molecules.[34,35] The second group (P-selectin, and IL6) comprised responses present in WT endothelia, but not in PTX3 KO endothelia. These are also proinflammatory genes that have demonstrated maladaptive effects during ischemic AKI.[41] The lesser expression of these genes by primary renal endothelia stimulated by TLR4 ligands in PTX3 KO mice may contribute to the lesser IRI seen in these mice. The third group (FGF2 and NOS3) comprised responses that were present in PTX3 KO but not WT mice. FGF2 ameliorates ischemic AKI,[30,31] and NOS3 should help preserve renal blood flow.[32,33]

DISCUSSION

We found that KO of PTX3 ameliorated IRI as assessed by both renal function and morphology. This is consistent with the detrimental effect of PTX3 on murine gastrointestinal IRI.[42,43]

This is, however, in contrast to the beneficial effect of PTX3 on murine cardiac IRI. Cardiac IRI exposes a non-muscle myosin heavy chain II. This is bound by a natural IgM autoantibody that activates complement the C1q-dependent classical pathway;[44] soluble PTX3 binds soluble C1q and inhibits such classical pathway activation.[45] In contrast, PTX3 has no effect on complement activation during renal IRI. Renal IRI does activate complement but not by natural autoantibodies and the classical pathway.[46,47] Instead, tubular injury decreases Crry, the murine homolog of human MCP (membrane cofactor protein) and DAF (decay accelerating factor); Crry normally prevents amplification of the alternative complement pathway after 'C3 tickover'.[48] In the absence of tubular Crry, alternative complement activation continues unrestrained and renal tubular injury results.[49,50] As shown by

others,[49] we saw more C3 activation on injured and dead tubules in WT kidneys after IRI than in PTX3 KO kidneys (Supplementary Figure S4 online). The C3 was not associated with endothelia (the location of PTX3). There was decreased C3 deposition on PTX3 KO tubules; this was secondary to the decreased tubular injury, not a direct effect of PTX3 KO.

Although PTX3 is expressed by endothelia, leukocytes, and other tissues,[6] we found PTX3 almost exclusively on endothelia in the kidney by immunohistology (Figure 4) and by the study of isolated endothelia and leukocytes (see Supplementary Figure S3A online).

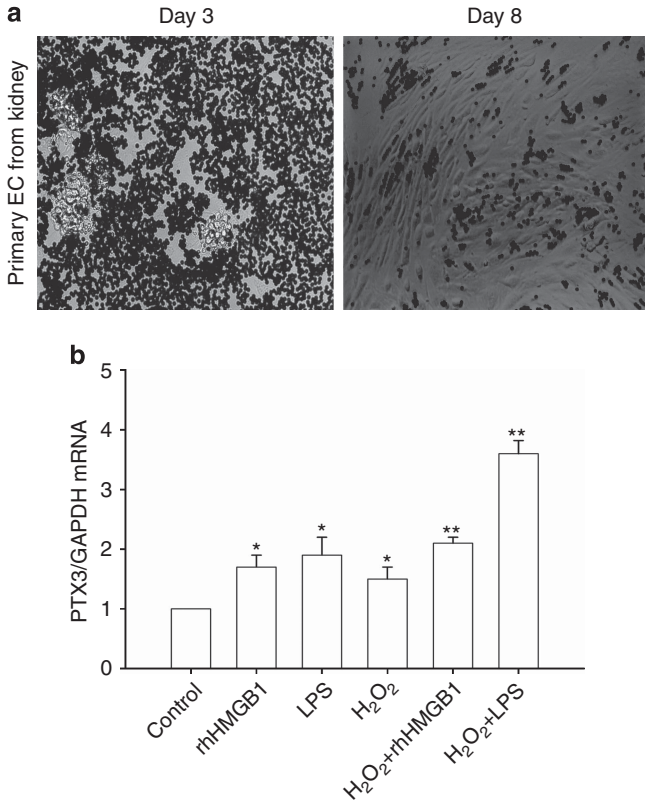

Figure 6 | Primary culture of renal endothelial cells (ECs). (**a**) Images of CD31+ Dynabead-isolated cells from kidney: primary ECs grown *in vitro* on day 3 and primary EC at confluence on day 8. Cells showed typical cobblestone morphology. (**b**) Pentraxin 3 (PTX3) messenger RNA (mRNA) on primary EC was upregulated *in vitro* by H_2O_2 (100 μmol/l), and/or recombinant human high-mobility group protein B1 (rhHMGB1; 5 μg/ml), and/or lipopolysaccharide (LPS; 5 μg/ml). Error bars show mean ± s.e.m., $n = 4$, $^*P < 0.05$, $^{**}P < 0.001$ vs. control.

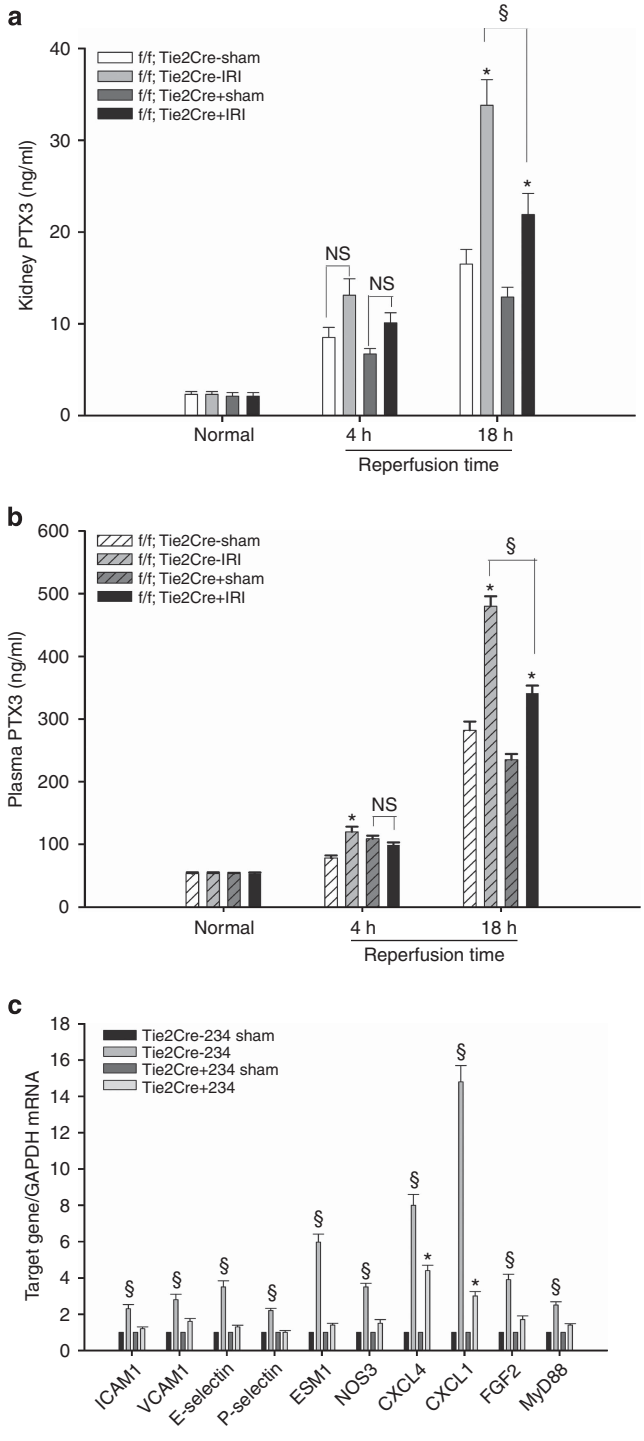

Figure 7 | Conditional endothelial knockout of MyD88 decreases pentraxin 3 (PTX3) production and endothelial activation. Renal pedicles of 'Wild-type' [MyD88f/f;Tie2Cre(−)] and 'conditional MyD88 KO' [MyD88f/f;Tie2Cre(+)] mice were clamped for 23 min. (**a**) Kidney PTX3 was measured by ELISA at baseline, 4-h, and 18-h reperfusion. The right kidney was harvested as control. (**b**) Plasma PTX3 was measured by ELISA at the same time points. In both **a** and **b**, the error bars stand for mean ± s.e.m., $n = 3$ per group. The four groups at each time point were analyzed by one-way analysis of variance, and then pairwise comparisons made by the Holm-Sidak method. $^*P < 0.05$ ischemia-reperfusion injury (IRI) vs. sham, $^{\S}P < 0.05$ IRI MyD88f/f;Tie2Cre(−) vs. MyD88f/f, Tie2Cre(+) at 18-h reperfusion; NS, not significant. (**c**) Endothelial markers were detected by quantitative reverse transcription PCR on CD31+ cells isolated from kidneys at 4-h reperfusion. Data were analyzed by the comparative *C*t method. The calibrator gene is the gene of interest taken from the sham kidney. Error bars stand for mean ± s.e.m., $n = 3$ per group, $^{\S}P < 0.05$ MyD88f/f;Tie2Cre(−) IRI vs. sham, $^*P < 0.05$ MyD88f/f;Tie2Cre(+) IRI vs. sham. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA.

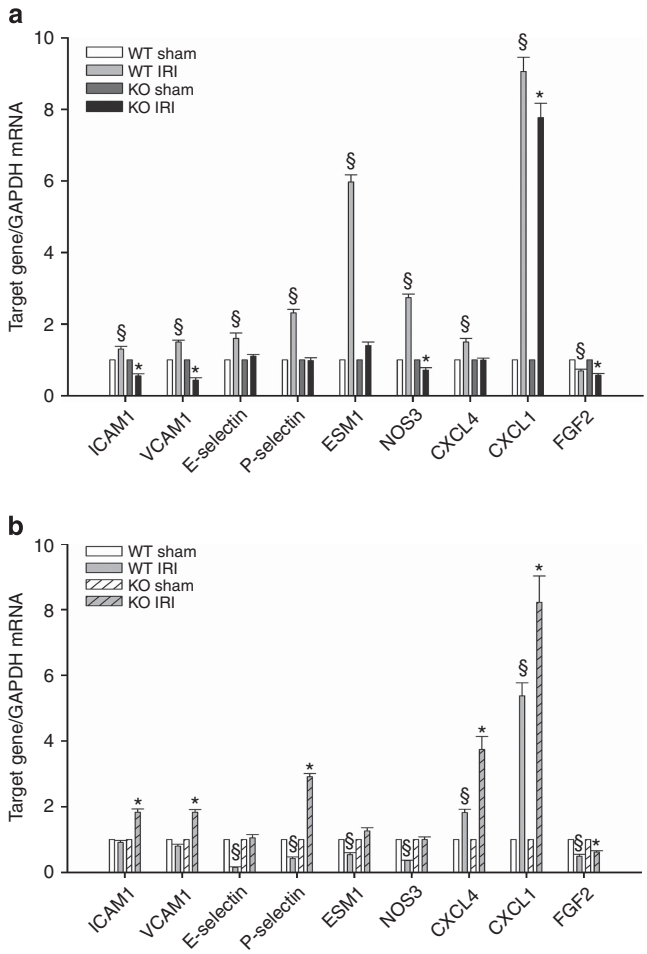

Figure 8 | CD31+ cells from pentraxin 3 (PTX3) KO ischemic kidneys show delayed endothelial activation. Renal pedicles of wild-type (WT) and PTX3 knockout (KO) mice were clamped for 23 min. CD31-bound Dynabeads were used to isolate endothelial cells from kidney digest at 4-h and 24-h reperfusion. Endothelial markers were detected by quantitative reverse transcription PCR (qRT-PCR) and data were analyzed by the comparative *C*t method. The calibrator gene is the gene of interest taken from the sham kidney. (**a**) 4-h reperfusion. (**b**) 24-h reperfusion. Error bars represent mean ± s.e.m., $n = 3$ per group, $^{\S}P < 0.05$ WT ischemia-reperfusion injury (IRI) vs. WT sham, $^{*}P < 0.05$ PTX3 KO IRI vs. KO sham. FGF2, fibroblast growth factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA.

Although we cannot exclude the possibility that some PTX3 is deposited in the kidney from the blood during ischemic AKI, the following data show that most of the increased endothelial PTX3 is produced by endothelial cells. (a) In Figure 3, the increased PTX3 messenger RNA in WT TLR4-sufficient ischemic kidneys is correlated with an increased PTX3 protein found on peritubular endothelia. This correlation is consistent with renal endothelial production of PTX3. (b) *In vitro* stimulation of endothelia by HMGB1 and ROS, both present in the ischemic kidney, activates PTX3 gene expression (Figures 5 and 6). (c) If PTX3 was extravasated from blood into the kidney, one would expect diffuse PTX3 staining because it binds to apoptotic cells,[51] DNA released by dying cells,[52] and extracellular complexes of tumor necrosis factor–stimulated gene 6/inter-alpha-trypsin inhibitor/hyaluronan.[53,54] The latter are found in the extracellular matrices of injured kidneys.[55,56] Instead, our immunostaining localized PTX3 protein to endothelia; this is consistent with its production by these cells. (d) Finally, our idea that PTX3 is produced locally in the kidney is consistent with the literature. The literature indicates that PTX3 is produced locally at sites of inflammation and then escapes into blood rather than vice versa; this is unlike the other major member of the pentraxin family, CRP, which is produced in the liver, enters the blood, and then extravasates into peripheral tissues.[57]

We explored both upstream events that regulate renal endothelial PTX3 as well as downstream events by which PTX3 regulates renal IRI.

Upstream, TLR4 was one important positive regulator of PTX3 because PTX3 expression *in vivo* was increased after IRI in WT, but not in TLR4-deficient, kidneys. HMGB1, released by injured cells during renal IRI, is the major ligand for TLR4. Inhibition of HMGB1 with specific antibodies ameliorates ischemic AKI.[58,59] Although HMGB1 binds to a number of different receptors,[15] TLR4 is the major maladaptive receptor during ischemic AKI. Thus, direct binding of HMGB1 to TLR4 has been demonstrated by biophysical techniques;[60] KO of RAGE, the other major receptor for HMGB1, has no effect on ischemic AKI;[61] in contrast, KO or inactivation of natural mutations of TLR4 in mice ameliorate ischemic AKI.[21,23,62,63] In addition, mutations that decrease TLR4 signaling in humans decrease ischemic AKI after transplantation.[64]

We found that recombinant HMGB1 increased PTX3 expression both in the MS1 endothelial cell line and WT renal endothelial primary cultures. Furthermore, we found that conditional MyD88 KO on endothelia decreased PTX3 protein from 33.8 ± 2.8 to 21.9 ± 2.3 ng/ml at 18-h reperfusion. The residual PTX3 expression might be due to incomplete KO of endothelial MyD88, or the MyD88-independent pathways of TLR4 signaling, or ROS released during IRI. Altogether, our data suggest that PTX3 is increased on endothelia mainly by HMGB1 acting on TLR4 via the MyD88 signaling pathway.

We also made the important observation that partial conditional KO of endothelial MyD88 markedly decreased the expression of endothelial adhesion molecules after renal IRI; this confirms our previous observation that endothelial TLR4 is required for endothelial activation,[5] and shows that this is due, in large part, to the MyD88 pathway.

In addition, we found that ROS produced during IRI[16–18] also contributes to the increased PTX3 expression. ROS synergize with the TLR4 ligands, HMGB1 or LPS, to increase PTX3 expression. To our knowledge, ROS has not previously been reported to regulate PTX3.

Downstream, we used two different experimental systems to study the effects of PTX3 on endothelial activation during ischemic AKI.

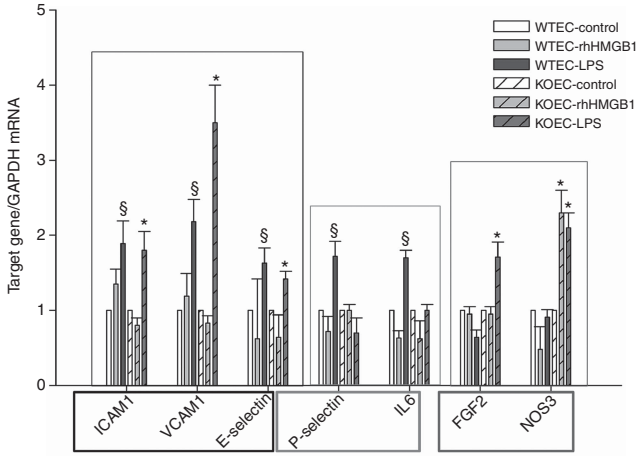

Figure 9 | Fundamental differences in primary cultures of endothelial cells from wild-type (WT) and pentraxin 3 (PTX3) knockout (KO) kidneys. Primary renal endothelial culture (EC) was maintained in Clonetics EGM-2 endothelial cell growth medium-2. Cells were treated with recombinant human high-mobility group protein B1 (rhHMGB1) or lipopolysaccharide (LPS) at 5 μg/ml for 4 h in culture. RNA was extracted and quantitative reverse transcription PCR performed. Error bars show mean mean ± s.e.m., $n = 3$, $^{\S}P < 0.05$ WT treated vs. control, $^{*}P < 0.05$ PTX3 KO treated vs. control. FGF2, fibroblast growth factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA.

(1) We isolated endothelia from ischemic WT and PTX KO kidneys. KO of PTX3 prevents the early expression of endothelial adhesion molecules and chemokines known to facilitate early maladaptive inflammation.[65–69] Altogether, these data suggest that increased PTX3 is one mediator of early maladaptive endothelial activation triggered by TLR4 that we previously reported.[5] In contrast to the decreased endothelial adhesion molecule expression at 4 h caused by PTX3 KO, we found increased expression of these adhesion molecules compared with WT at 24 h after IRI. This should facilitate the late influx of leukocytes that participate in tissue repair and turn off the inflammatory response.[36–40] Thus, our results show that PTX3 KO had different effects at 4-h reperfusion compared with 24-h reperfusion.

(2) In addition, we also studied primary cultures of renal endothelia from unmanipulated WT and PTX KO mice. After stimulation by TLR4 ligands, the responses of the WT and PTX3 KO primary endothelia could be divided into three groups. Some activation functions were the same. Expression of other proinflammatory adhesion molecules required PTX3. Expression of some endothelial functions, such as FGF2 and NOS3, which should protect or repair the ischemic kidney, was increased in the PTX3 KO endothelia.

Each of our two experimental systems has advantages and disadvantages. The endothelial cells isolated from ischemic kidneys receive the entire spectrum of signals from the ischemic environment, but subpopulations may be selected and functions may be altered by the enzymatic and mechanical disruption during isolation. The endothelia grown in primary culture avoid the above problems, but the artificial extracellular matrix and growth factors (VEGF, FGF, IGF1, HGF, hydrocortisone, and heparin) may not fully replicate the microenvironment *in vivo*.

The main point is that both systems show that PTX3 KO inhibits maladaptive endothelial responses to TLR4 ligands. The 'minor' differences between the two systems may result from the following: *in vivo*, no intracellular signaling pathway, including that for TLR4, operates in isolation, and any change in cell functions integrates the 'cross talk' between multiple simultaneous signaling pathways.[70–72] In other words, renal endothelial cells *in vivo* receive signals from a variety of growth factors and cytokines during AKI in addition to TLR4 ligands. Given this complexity, the profound anti-inflammatory effects of KO of single pathway (PTX3) are remarkable both *in vivo* (Figure 8) and after stimulation *in vitro* (Figure 9). The differences in a few details are likely the result of 'cross talk' between TLR4 and other signaling pathways, which are different after stimulation *in vivo* and *in vitro*, and will be the focus of future studies. These differences include the increase in adaptive FGF2 and NOS3 seen after *in vitro* stimulation (Figure 9), but not in endothelia isolated from ischemic kidneys (Figure 8).

In addition, although HMGB1 is a major and the best studied TLR4 ligand released during ischemic AKI,[21,23,58–60,62,63] other TLR4 ligands such as stress fibronectin[73] are expected to be produced and may activate endothelial TLR4 during ischemic AKI *in vivo*. Each TLR4 ligand may elicit a slightly different response,[74,75] and contribute to the 'cross talk' discussed above. This is illustrated by the different responses to HMGB1 and endotoxin in Figure 9.

We suggest that PTX3 may exacerbate the above-mentioned endothelial stress responses during renal IRI by binding and inhibiting growth factors that would otherwise ameliorate endothelial injury and promote repair. The N-terminus of PTX3 specifically binds and inhibits FGF8[76] and FGF2.[77,78] Such inhibition would have detrimental effects on renal endothelia because FGF2 ameliorates rodent ischemic AKI.[30,31]

Another possibility is that PTX3 increases the exposure of renal endothelial cells to proinflammatory cytokines by inhibiting 'efferocytosis', which is defined as the phagocytosis of apoptotic cells and which does not result in the release of proinflammatory cytokines. Apoptosis is a major type of cell death during ischemic AKI (see review[79]). PTX3 inhibits efferocytosis;[80–83] such inhibition of apoptotic renal cells would allow these apoptotic cells to degenerate (die a post-apoptotic death) and release proinflammatory damage–associated molecular pattern molecules.[84–86] Consistent with this formulation is the increased necrosis and inflammation we observed in the WT compared with the PTX3 KO ischemic kidneys. The idea that efferocytosis inhibits the maladaptive inflammatory response to renal IRI is supported by the beneficial effects of increasing efferocytosis by injections of MFG-E8 (milk fat globule-EGF factor 8/lactadherin);[87,88] MFG-E8 is a 'bridging molecule' that links

the apoptotic cells to phagocytes, and thus increases efferocytosis.[89]

In summary, PTX3 may have fundamental importance for biology and disease because it has been so conserved during evolution.[6] We have now demonstrated important links between PTX3 and the renal endothelium, TLR4, MyD88, and ROS in the pathogenesis of ischemic IRI. We showed that PTX3 KO ameliorates ischemic AKI. PTX3 expression is increased via ROS, and a MyD88-dependent TLR4-mediated mechanism on renal endothelia. PTX3 is required for the early expression of endothelial adhesion molecules and chemokines that facilitate the maladaptive inflammatory response to IRI. PTX3 inhibits the late endothelial expression of adhesion molecules that may facilitate immigration of regulatory and reparative leukocytes.

MATERIALS AND METHODS

Mice

Six- to eight-week-old male C57BL/10, TLR4-null mice C57BL/10ScNJ, MyD88-floxed mice B6.129P2(SJL)-*Myd88*^{tm1Defr}/J, and Tie2 Cre 'driver' B6.Cg-Tg(Tek-cre)1Ywa/J mice were from The Jackson Laboratory (Bar Harbor, ME). To avoid non-cell-specific deletion of floxed alles arising from germ line Cre-recombinase activity,[90] female MyD88f/f mice were interbred with male MyD88f/f; Tie2Cre(+) mice to generate conditional endothelial cell–specific KO MyD88 mice (MyD88f/f;Tie2Cre+) and littermate control mice (MyD88f/f; Tie2Cre−). PTX3 KO founders were obtained from Dr Martin M. Matzuk (Baylor College of Medicine, Houston, TX). Because of the subfertility of female PTX3 KO mice,[91] we bred heterozygous male and heterozygous female mice. Littermates of PTX3+/+ were used as controls. Genomic DNA from offspring was extracted using the Extract-N-Amp Tissue PCR kit (Sigma, Saint Louis, MO). Primers for genotyping were described in detail in Supplementary Table S1 online.

Renal IRI

After right nephrectomy, the left renal pedicle was occluded for either 16 or 23 min; sham-operated mice were used as controls.[5] All mouse work was approved by the UT Southwestern Institutional Animal Care and Use Committee.

DNA microarray

Detailed methods can be found in the Supplementary Materials online.

Renal function

Scr was measured using the P/ACE MDQ Capillary Electrophoresis System with a PDA Detector (Beckman Coulter, Indianapolis, IN).[9] Blood urea nitrogen was measured using the VITROS BUN slides on VITROS 250 Chemistry Analyzer (Ortho Clinical Diagnostics, Raritan, NJ).

Histology and immunohistology

For histology, kidneys were harvested at 24-h reperfusion and fixed in 10% neutral-buffered formalin (Sigma). Paraffin-embedded sections were stained with hematoxylin and eosin and anti-myeloperoxidase (rabbit anti-myeloperoxidase polyclonal antibody, Thermo Scientific, Rockford, IL). Tissue damage and inflammation was evaluated as previously reported.[53] For immunofluorescence staining, mice were *in situ* perfused with cold PBS followed by cold 4% PFA. Detailed sample processing can be found in our previous report.[5] Sections were blocked with 10% goat serum and stained with rat anti-mouse Pentraxin 3 mAb (clone 265629, R&D Systems, Minneapolis, MN) or rat anti-mouse Complement component 3 mAb (clone RmC11H9, Cedarlane Laboratories, Burlington, NC). Rat IgG2b or IgG2a was used as isotype control. For double staining of CD31 and PTX3, rat anti-mouse CD31 mAb (clone MEC13.3, BD Pharmingen, San Diego, CA) was applied to the section followed by Texas Red–conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human PTX3 pAb (which cross reacts with mouse and rat, Santa Cruz Biotechnology), and then fluorescein goat anti-rabbit IgG (Santa Cruz Biotechnology). Sections were mounted with VECTASHIELD Mounting Medium with 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and visualized using a Carl Zeiss Axioplan2 Imaging microscope (Carl Zeiss MicroImaging, Thornwood, NY). Each staining was carried out on four individual mice per group and the images in the figures are representative of each group.

Isolation of endothelial cells and leukocytes

Detailed procedure can be found in our previous reports.[5,53]

Primary renal endothelial cell culture

Bead-bound CD31+ cells isolated from kidneys were resuspended in Clonetics EGM-2 endothelial cell growth medium-2 BulletKit (Lonza, Allendale, NJ) and plated into BD BioCoat Fibronectin 24-well Multiwell plate (BD Pharmingen). Medium was changed every 3 days. Primary culture passage 1 cells were used for experiment.

Hydrogen peroxide (H_2O_2), LPS, rhHMGB1 treatment

Confluent MS1 monolayers or primary renal endothelial cells were washed with Dulbecco's phosphate-buffered saline three times. Cells were exposed to H_2O_2 (Sigma) at 100 μmol/l for 30 min in Earle's balanced salt solution at 37 °C, then replenished with complete medium containing 10% fetal calf serum. Some cells were treated with rhHMGB1 (R&D Systems) at 5 μg/ml or with an ultrapure form of LPS that activates only TLR4 (*Escherichia coli* 0111:B4, InvivoGen, San Diego, CA) for an additional 4 h, followed by RNA extraction using the RNeasy Mini kit (Qiagen, Valencia, CA).

Real-time reverse transcription PCR

Detailed procedure can be found in our previous reports.[5,53] PCR primers are listed in Supplementary Table S2 online.

Enzyme-linked immunosorbent assay (ELISA)

Blood and kidney samples were collected at 4, 18, 24 h, day 2, day 3, day 5, and day 7 reperfusion. Plasma was obtained by centrifuging blood samples at 2000 × *g* for 15 min at 4 °C. Kidneys were homogenized in RIPA buffer supplemented with Protease inhibitor cocktail (Sigma) on ice. Samples were centrifuged at 12,000 r.p.m. for 15 min at 4 °C. The protein concentration was measured by Coomassie Plus (Bradford) Assay Reagent (Thermo Scientific) in triplicate. All samples were stored at −80 °C until analysis. PTX3 protein of each sample was assayed in duplicate using Mouse Pentraxin 3 Quantikine ELISA Kits (R&D Systems) according to the manufacturer's instructions. Absorbance was read on Pelkin Elmer 1420 Multilabel Counter Victor3 plate reader (Pelkin-Elmer, Waltham, MA) at 450 nm, with correction wavelength set at 540 nm.

Western blot analysis

Protein extracts were separated by 10% precast SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), probed with goat anti-mouse C3 pAb (ICL, Portland, OR) or rat anti-mouse C3 mAb (RMC11H9, Cdarlane Labs), exposed to a horseradish peroxidase-conjugated secondary antibody (Sigma), and visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the supplier's instructions. The beta Actin Antibody (horseradish peroxidase; GenScript, Piscataway, NJ) was used as loading control.

Statistics

The data were presented as mean ± s.e.m. One-way analysis of variance Holm-Sidak method and two-tailed Student's *t*-test were carried out using SigmaPlot 11.0. Differences with a P value < 0.05 were considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used for mouse genotyping.
Table S2. Primers used for real-time RT-PCR.
Figure S1. PTX3 antibody specificity on IRI kidney sections.
Figure S2. Double-staining of sham kidney sections shows the co-localization of PTX3 and CD31 on peritubular structures in the OM.
Figure S3. CD45+ leukocytes express PTX3.
Figure S4. Increased C3 in WT ischemic kidneys.
Figure S5. PTX3 co-localizes with CD31+ cells on kidney. These are high resolution photomicrographs shown at a lower resolution in Figure 4 of main manuscript.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Hsu CY, McCulloch CE, Fan D *et al.* Community-based incidence of acute renal failure. *Kidney Int* 2007; **72**: 208–212.
2. Coca SG, Singanamala S, Parikh CR. Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis. *Kidney Int* 2012; **81**: 442–448.
3. Venkatachalam MA, Griffin KA, Lan R *et al.* Acute kidney injury: a springboard for progression in chronic kidney disease. *Am J Physiol Renal Physiol* 2010; **298**: F1078.
4. Rosin DL, Okusa MD. Dangers within: DAMP responses to damage and cell death in kidney disease. *J Am Soc Nephrol* 2011; **22**: 416–425.
5. Chen J, John R, Richardson JA *et al.* Toll-like receptor 4 regulates early endothelial activation during ischemic acute kidney injury. *Kidney Int* 2011; **79**: 288–299.
6. Inforzato A, Jaillon S, Moalli F *et al.* The long pentraxin PTX3 at the crossroads between innate immunity and tissue remodelling. *Tissue Antigens* 2011; **77**: 271–282.
7. Kume N, Mitsuoka H, Hayashida K *et al.* Pentraxin 3 as a biomarker for acute coronary syndrome: comparison with biomarkers for cardiac damage. *J Cardiol* 2011; **58**: 38–45.
8. Ryu WS, Kim CK, Kim BJ *et al.* Pentraxin 3: a novel and independent prognostic marker in ischemic stroke. *Atherosclerosis* 2012; **220**: 581–586.
9. Zinellu A, Caria MA, Tavera C *et al.* Plasma creatinine and creatine quantification by capillary electrophoresis diode array detector. *Anal Biochem* 2005; **342**: 186–193.
10. Yuen PS, Dunn SR, Miyaji T *et al.* A simplified method for HPLC determination of creatinine in mouse serum. *Am J Physiol Renal Physiol* 2004; **286**: F1116–F11F9.
11. Dunn SR, Qi Z, Bottinger EP *et al.* Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* 2004; **65**: 1959–1967.
12. Waikar SS, Betensky RA, Emerson SC *et al.* Imperfect gold standards for kidney injury biomarker evaluation. *J Am Soc Nephrol* 2012; **23**: 13–21.
13. Horbelt M, Lee SY, Mang HE *et al.* Acute and chronic microvascular alterations in a mouse model of ischemic acute kidney injury. *Am J Physiol Renal Physiol* 2007; **293**: F688–F695.
14. Sutton TA, Mang HE, Campos SB *et al.* Injury of the renal microvascular endothelium alters barrier function after ischemia. *Am J Physiol Renal Physiol* 2003; **285**: F191–F198.
15. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol* 2011; **29**: 139–162.
16. Szeto HH, Liu S, Soong Y *et al.* Mitochodria-targeted peptide accelerates ATP recovery and reduces ischemic kidney injury. *J Am Soc Nephrol* 2011; **22**: 1041–1052.
17. Chatterjee PK. Novel pharmacological approaches to the treatment of renal ischemia-reperfusion injury: a comprehensive review. *Naunyn-Schmiedebergs Arch Pharmacol [Review]* 2007; **376**: 1–43.
18. Hall AM. Pores for thought: new strategies to re-energize stressed mitochondria in acute kidney injury. *J Am Soc Nephrol* 2011; **22**: 986–989.
19. Arbiser JL, Moses MA, Fernandez CA *et al.* Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. *Proc Natl Acad Sci USA* 1997; **94**: 861–866.
20. Arbiser JL, Larsson H, Claesson-Welsh L *et al.* Overexpression of VEGF 121 in immortalized endothelial cells causes conversion to slowly growing angiosarcoma and high level expression of the VEGF receptors VEGFR-1 and VEGFR-2 *in vivo*. *Am J Pathol* 2000; **156**: 1469–1476.
21. Wu H, Chen G, Wyburn KR *et al.* TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest* 2007; **117**: 2847–2859.
22. Shigeoka AA, Holscher TD, King AJ *et al.* TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J Immunol* 2007; **178**: 6252–6258.
23. Pulskens WP, Teske GJ, Butter LM *et al.* Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PLoS ONE* 2008; **3**: e3596.
24. Hou B, Reizis B, DeFranco AL. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. *Immunity* 2008; **29**: 272–282.
25. Schlaeger TM, Bartunkova S, Lawitts JA *et al.* Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci USA* 1997; **94**: 3058–3063.
26. Kisanuki YY, Hammer RE, Miyazaki J *et al.* Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis *in vivo*. *Dev Biol* 2001; **230**: 230–242.
27. O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* 2008; **226**: 10–18.
28. Hauser P, Kainz A, Perco P *et al.* Transcriptional response in the unaffected kidney after contralateral hydronephrosis or nephrectomy. *Kidney Int* 2005; **68**: 2497–2507.
29. Sarrazin S, Adam E, Lyon M *et al.* Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. *Biochim Biophys Acta* 2006; **1765**: 25–37.
30. Villanueva S, Cespedes C, Gonzalez A *et al.* bFGF induces an earlier expression of nephrogenic proteins after ischemic acute renal failure. *Am J Physiol Regul Integr Comp Physiol* 2006; **291**: R1677–R1687.
31. Villanueva S, Cespedes C, Gonzalez AA *et al.* Inhibition of bFGF-receptor type 2 increases kidney damage and suppresses nephrogenic protein expression after ischemic acute renal failure. *Am J Physiol Regul Integr Comp Physiol* 2008; **294**: R819–R828.
32. Goligorsky MS, Brodsky SV, Noiri E. Nitric oxide in acute renal failure: NOS versus NOS. *Kidney Int* 2002; **61**: 855–861.
33. Legrand M, Mik EG, Johannes T *et al.* Renal hypoxia and dysoxia after reperfusion of the ischemic kidney. *Mol Med* 2008; **14**: 502–516.
34. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? *Kidney Int* 2004; **66**: 480–485.
35. Jang HR, Rabb H. The innate immune response in ischemic acute kidney injury. *Clin Immunol* 2009; **130**: 41–50.
36. Lin SL, Li B, Rao S *et al.* Macrophage Wnt7b is critical for kidney repair and regeneration. *Proc Natl Acad Sci USA* 2010; **107**: 4194–4199.

37. Lee S, Huen S, Nishio H *et al.* Distinct macrophage phenotypes contribute to kidney injury and repair. *J Am Soc Nephrol* 2011; **22**: 317–326.
38. Gandolfo MT, Jang HR, Bagnasco SM *et al.* Mycophenolate mofetil modifies kidney tubular injury and Foxp3+ regulatory T cell trafficking during recovery from experimental ischemia-reperfusion. *Transpl Immunol* 2010; **23**: 45–52.
39. Kinsey GR, Huang L, Vergis AL *et al.* Regulatory T cells contribute to the protective effect of ischemic preconditioning in the kidney. *Kidney Int* 2010; **77**: 771–780.
40. Lai LW, Yong KC, Lien YH. Pharmacologic recruitment of regulatory T cells as a therapy for ischemic acute kidney injury. *Kidney Int* 2012; **81**: 983–992.
41. Kielar ML, John R, Bennett M *et al.* Maladaptive role of IL-6 in ischemic acute renal failure. *J Am Soc Nephrol* 2005; **16**: 3315–3325.
42. Souza DG, Soares AC, Pinho V *et al.* Increased mortality and inflammation in tumor necrosis factor-stimulated gene-14 transgenic mice after ischemia and reperfusion injury. *Am J Pathol* 2002; **160**(bett5): 1755–1765.
43. Souza DG, Amaral FA, Fagundes CT *et al.* The long pentraxin PTX3 is crucial for tissue inflammation after intestinal ischemia and reperfusion in mice. *Am J Pathol* 2009; **174**: 1309–1318.
44. Haas MS, Alicot EM, Schuerpf F *et al.* Blockade of self-reactive IgM significantly reduces injury in a murine model of acute myocardial infarction. *Cardiovasc Res* 2010; **87**: 618–627.
45. Salio M, Chimenti S, De Angelis N *et al.* Cardioprotective function of the long pentraxin PTX3 in acute myocardial infarction. *Circulation* 2008; **117**: 1055–1064.
46. Renner B, Strassheim D, Amura CR *et al.* B cell subsets contribute to renal injury and renal protection after ischemia/reperfusion. *J Immunol* 2010; **185**: 4393–4400.
47. Zhou W, Farrar CA, Abe K *et al.* Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 2000; **105**: 1363–1371.
48. Ricklin D, Hajishengallis G, Yang K *et al.* Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010; **11**: 785–797.
49. Thurman JM, Renner B. Dynamic control of the complement system by modulated expression of regulatory proteins. *Lab Invest* 2011; **91**: 4–11.
50. Bao L, Wang Y, Chang A *et al.* Unrestricted C3 activation occurs in Crry-deficient kidneys and rapidly leads to chronic renal failure. *J Am Soc Nephrol* 2007; **18**: 811–822.
51. Deban L, Jarva H, Lehtinen MJ *et al.* Binding of the long pentraxin PTX3 to factor H: interacting domains and Function in the regulation of complement activation. *J Immunol* 2008; **181**: 8433–8440.
52. Jaillon S, Peri G, Delneste Y *et al.* The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med* 2007; **204**: 793–804.
53. Salustri A, Garlanda C, Hirsch E *et al.* PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in *in vivo* fertilization. *Development* 2004; **131**: 1577–1586.
54. Scarchilli L, Camaioni A, Bottazzi B *et al.* PTX3 interacts with inter-alpha-trypsin inhibitor: implications for hyaluronan organization and cumulus oophorus expansion. *J Biol Chem* 2007; **282**: 30161–30170.
55. Janssen U, Thomas G, Glant T *et al.* Expression of inter-alpha-trypsin inhibitor and tumor necrosis factor-stimulated gene 6 in renal proximal tubular epithelial cells. *Kidney Int* 2001; **60**: 126–136.
56. Bommaya G, Meran S, Krupa A *et al.* Tumour necrosis factor-stimulated gene (TSG)-6 controls epithelial-mesenchymal transition of proximal tubular epithelial cells. *Int J Biochem Cell Biol* 2011; **43**: 1739–1746.
57. Bottazzi B, Garlanda C, Cotena A *et al.* The long pentraxin PTX3 as a prototypic humoral pattern recognition receptor: interplay with cellular innate immunity. *Immunol Rev* 2009; **227**: 9–18.
58. Wu H, Ma J, Wang P *et al.* HMGB1 contributes to kidney ischemia reperfusion injury. *J Am Soc Nephrol* 2010; **21**: 1878–1890.
59. Li J, Gong Q, Zhong S *et al.* Neutralization of the extracellular HMGB1 released by ischaemic damaged renal cells protects against renal ischaemia-reperfusion injury. *Nephrol Dial Transplant* 2010; **26**: 469–478.
60. Yang H, Hreggvidsdottir HS, Palmblad K *et al.* A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci USA* 2010; **107**: 11942–11947.
61. Dessing MC, Pulskens WP, Teske GJ *et al.* RAGE does not contribute to renal injury and damage upon ischemia/reperfusion-induced injury. *J Innate Immun* 2012; **4**: 80–85.
62. Rusai K, Sollinger D, Baumann M *et al.* Toll-like receptors 2 and 4 in renal ischemia/reperfusion injury. *Pediatr Nephrol* 2010; **25**: 853–860.
63. Chen J, Hartono J, John R *et al.* Interleukin 6 production by leukocytes during ischemic acute kidney injury is regulated by TLR4. *Kidney Int* 2011; **80**: 504–515.
64. Kruger B, Krick S, Dhillon N *et al.* Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. *Proc Natl Acad Sci USA* 2009; **106**: 3390–3395.
65. Kelly KJ, Williams Jr WW, Colvin RB *et al.* Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 1996; **97**: 1056–1063.
66. Kelly KJ, Williams Jr WW, Colvin RB *et al.* Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. *Proc Natl Acad Sci USA* 1995; **91**: 812–816.
67. Rabb H, Mendiola CC, Dietz J *et al.* Role of CD11a and CD11b in ischemic acute renal failure in rats. *Am J Physiol* 1994; **267**: F1052–F1058.
68. Kiew LV, Munavvar AS, Law CH *et al.* Effect of antisense oligodeoxynucleotides for ICAM-1 on renal ischaemia-reperfusion injury in the anaesthetised rat. *J Physiol* 2004; **557**(Part 3): 981–989.
69. Haller H, Dragun D, Miethke A *et al.* Antisense oligonucleotides for ICAM 1 attenuate reperfusion injury and renal failure in the rat. *Kidney Int* 1996; **50**: 473–480.
70. O'Neill LA. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity* 2008; **29**: 12–20.
71. Bezbradica JS, Medzhitov R. Integration of cytokine and heterologous receptor signaling pathways. *Nat Immunol* 2009; **10**: 333–339.
72. Ostuni R, Zanoni I, Granucci F. Deciphering the complexity of Toll-like receptor signaling. *Cell Mol Life Sci* 2010; **67**: 4109–4134.
73. You R, Zheng M, McKeown-Longo PJ. The first type III repeat in fibronectin activates an inflammatory pathway in dermal fibroblasts. *J Biol Chem* 2010; **285**: 36255–36259.
74. Mittal D, Saccheri F, Venereau E *et al.* TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. *EMBO J* 2010; **29**: 2242–2252.
75. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm* 2010; **2010**: 1–21.
76. Leali D, Alessi P, Coltrini D *et al.* Long pentraxin-3 inhibits FGF8b-dependent angiogenesis and growth of steroid hormone-regulated tumors. *Mol Cancer Ther* 2011; **10**: 1600–1610.
77. Leali D, Bianchi R, Bugatti A *et al.* Fibroblast growth factor 2-antagonist activity of a long-pentraxin 3-derived anti-angiogenic pentapeptide. *J Cell Mol Med* 2010; **14**: 2109–2121.
78. Rusnati M, Camozzi M, Moroni E *et al.* Selective recognition of fibroblast growth factor-2 by the long pentraxin PTX3 inhibits angiogenesis. *Blood* 2004; **104**: 92–99.
79. Havasi A, Borkan SC. Apoptosis and acute kidney injury. *Kidney Int* 2011; **80**: 29–40.
80. Rovere P, Peri G, Fazzini F *et al.* The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. *Blood* 2000; **96**: 4300–4306.
81. Baruah P, Dumitriu IE, Peri G *et al.* The tissue pentraxin PTX3 limits C1q-mediated complement activation and phagocytosis of apoptotic cells by dendritic cells. *J Leukoc Biol* 2006; **80**: 87–95.
82. van Rossum AP, Fazzini F, Limburg PC *et al.* The prototypic tissue pentraxin PTX3, in contrast to the short pentraxin serum amyloid P, inhibits phagocytosis of late apoptotic neutrophils by macrophages. *Arthritis Rheum* 2004; **50**: 2667–2674.
83. van Rossum AP, Pas HH, Fazzini F *et al.* Abundance of the long pentraxin PTX3 at sites of leukocytoclastic lesions in patients with small-vessel vasculitis. *Arthritis Rheum* 2006; **54**: 986–991.
84. Poon IK, Hulett MD, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ* 2010; **17**: 381–397.
85. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* 2010; **10**: 36–46.
86. Peter C, Wesselborg S, Herrmann M *et al.* Dangerous attraction: phagocyte recruitment and danger signals of apoptotic and necrotic cells. *Apoptosis* 2010; **15**: 1007–1028.
87. Matsuda A, Wu R, Jacob A *et al.* Protective effect of milk fat globule-epidermal growth factor-factor VIII after renal ischemia-reperfusion injury in mice. *Crit Care Med* 2011; **39**: 2039–2047.
88. Harrois A, Duranteau J. Acute kidney injury: clear the kidney of apoptotic debris!. *Crit Care Med* 2011; **39**: 2180–2181.
89. Matsuda A, Jacob A, Wu R *et al.* Milk fat globule-EGF factor VIII in sepsis and ischemia-reperfusion injury. *Mol Med* 2011; **17**: 126–133.
90. de Lange WJ, Halabi CM, Beyer AM *et al.* Germ line activation of the Tie2 and SMMHC promoters causes noncell-specific deletion of floxed alleles. *Physiol Genomics* 2008; **35**: 1–4.
91. Varani S, Elvin JA, Yan C *et al.* Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Mol Endocrinol* 2002; **16**: 1154–1167.